Immunity, Vol. 3, 175–186, August, 1995, Copyright © 1995 by Cell Press

Tissue-Regulated Differentiation and Maturation of a v-*abl*-Immortalized Mast Cell-Committed Progenitor

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Summary

An immature v-*abl*-transformed mast cell line (V3-MC) was derived from a mouse that developed systemic mastocytosis after transplantation of v-*abl*-infected bone marrow cells. V3-MCs injected intravenously into adult BALB/c mice infiltrated the liver, spleen, and intestine by day 6 and underwent progressive differentiation and maturation, eventually resembling indigenous mast cells. In terms of their protease content, the V3-MCs that localized in the liver and spleen differed from those in the intestine, and both differed from the cultured V3-MCs. The acquired expression of certain proteases and the loss of expression of other proteases in these tissue V3-MCs defines particular phenotypes and indicates that the differentiation and maturation of mast cell-committed progenitor cells are primarily regulated by factors in the different tissue microenvironments.

Introduction

Mast cells consist of a heterogeneous family of immune effector cells that exhibit subclass-specific functional responses. Despite the involvement of mast cells in various biologic processes, information about how their differentiation and maturation are regulated is just beginning to emerge. Mast cells are derived primarily from multipotential stem cells in the bone marrow (Kitamura et al., 1978; Nakahata et al., 1982; Schrader et al., 1981; Sonoda et al., 1982; Ihle et al., 1983; Yung et al., 1983; Nakano et al., 1985; Jarboe et al., 1989; Leftwich et al., 1992). However, unlike other hematopoietic cell types, mature mast cells are not found in the peripheral blood. Thus, it has been proposed that mast cells complete their development only when they migrate into specific tissues.

One scheme to classify mast cells is based on the composition of their granules. Neutral proteases account for the vast majority of granule protein in mast cells and, over the last few years, eight different mouse mast cell proteases have been identified, cloned, or both (reviewed by Hunt and Stevens, 1995). The pattern of expression of the neutral proteases varies among the mast cells found in different tissues. For example, in the BALB/c mouse, the mast cells that populate the skin express mouse mast cell protease (mMCP) 4, mMCP-5, mMCP-6, mMCP-7, and carboxypeptidase A (mMC-CPA) (Stevens et al., 1994). In contrast, the mast cells that populate the intestine of the helminth-infected BALB/c mouse preferentially express mMCP-1 (Le Trong et al., 1989) and mMCP-2 (Serafin et al., 1990). To account for the heterogeneity observed in tissue mast cells, either distinct populations of mast cell–committed progenitors must exist in the bone marrow that are each programmed to express a specific panel of proteins or the final stages of mast cell differentiation and maturation must be controlled by the tissue microenvironment.

One approach to understand the factors that regulate the development of mast cells in vivo has been to study the effects of different cytokines on cultured mast cells. Immature nontransformed bone marrow–derived mast cells (mBMMCs) have been obtained by culturing bone marrow cells from BALB/c mice for 2–3 weeks in medium containing interleukin-3 (IL-3) (Ihle et al., 1983). The proteoglycan content and/or neutral granule protease content of these mBMMCs can be modulated by subsequent coculture with fibroblasts (Levi-Schaffer et al., 1986) or culture in medium containing various combinations of IL-3, IL-4, IL-9, IL-10, and c-*kit* ligand (Tsai et al., 1991; Gurish et al., 1992a; Ghildyal et al., 1992a, 1993; Eklund et al., 1993). However, these in vitro systems have the disadvantage that many of the mast cells obtained possess granule phenotypes that are different from known populations of tissue mast cells. An alternative approach to study mast cell differentiation and maturation has been to immortalize mast cell progenitors by transformation with retroviral oncogenes. Immortalized mast cell lines have been obtained by in vitro infection of hematopoietic cells with Abelson murine leukemia virus (Pierce et al., 1985), Harvey sarcoma virus (Rein et al., 1985), or Kirsten sarcoma virus (Reynolds et al., 1988). Although these cell lines have proved important for the identification of mast cell–specific proteins and genes, their continuous release of infectious retrovirus complicates their usefulness for the study of tissue-directed pleiotropism in vivo.

Techniques permitting helper-free retrovirus production offer a potential solution to the problem that hindered the in vivo use of immortalized mast cell lines. Using these techniques, we have generated mast cell lines (designated V3-MC and V7-MC) from BALB/c mice that developed systemic mastocytosis after lethal irradiation and transplantation with bone marrow infected by high titer helper-free

[6]Drs. Gurish and Pear contributed equally to this study.

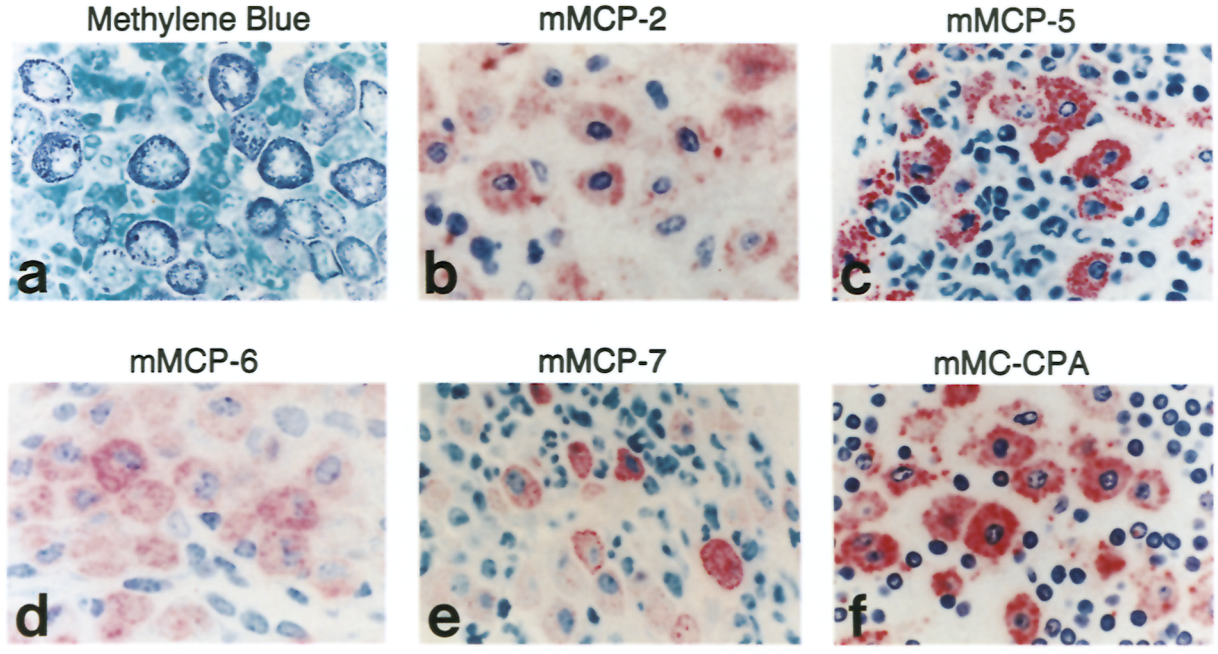


Figure 1. Histochemical and Immunohistochemical Analysis of the Spleen from a Primary Mastocytosis Mouse

Sections of a spleen from an irradiated BALB/c mouse reconstituted with v-*abl*-transduced bone marrow cells were evaluated histochemically with methylene blue (a) or immunohistochemically with anti-mMCP-2 immunoglobulin (b), anti-mMCP-5 immunoglobulin (c), anti-mMCP-6 immunoglobulin (d), anti-mMCP-7 immunoglobulin (e), or anti-mMC-CPA immunoglobulin (f). The findings were similar in the other three primary mastocytosis mice that were analyzed.

v-*abl*-expressing retrovirus. These mast cell lines are similar to IL-3-dependent BALB/c mBMMC in terms of their granularity and protease phenotype, and the intravenous injection of one of these cell lines into BALB/c mice results in a systemic mastocytosis. Examination of the mast cell phenotypes in several tissues shows that the injected V3-MCs acquire the neutral protease phenotype of the indigenous mast cells of a particular tissue. This finding supports a model in which the tissue microenvironment controls the final stages of mast cell development.

Results

Development of Mastocytosis in Lethally Irradiated Syngeneic Mice after the Adoptive Transfer of pGD$^{v\text{-}abl}$-Transduced Bone Marrow Cells

To evaluate the transforming capacity of a high titer helper-free v-*abl*-expressing retrovirus, 10 lethally irradiated BALB/c mice were injected intravenously with bone marrow that had been infected in vitro by pGD$^{v\text{-}abl}$. As assessed by polymerase chain reaction (PCR) analysis, 3 weeks after the injection of pGD$^{v\text{-}abl}$-transduced bone marrow cells, all mice contained cells with proviral DNA in their blood (data not shown). These mice showed evidence of cachexia, decreased activity, and decreased grooming 4–7 weeks after adoptive transfer. The white blood cell counts of 9 of the mice were similar to those of control animals, but one mouse had a markedly elevated white blood cell count due to leukemic blasts. The mice were sacrificed when they showed evidence of disease, and all had hepatosplenomegaly and lymphadenopathy. Histologic analysis revealed that the liver, spleen (Figure 1), lymph nodes, and bone marrow were populated by large numbers of granulated mast cells. In addition to mastocytosis, 5 of the mice had foci composed of immature blasts in the liver, spleen, lymph nodes, and bone marrow.

The histochemistry and immunohistochemistry of the spleen of one of the original mastocytosis mice is illustrated in Figure 1. Based on the metachromasia obtained after staining with methylene blue or toluidine blue, the spleen of this mastocytosis mouse contained >100 mast cells/mm^2. The infiltrating mast cells were 12–20 μm in diameter, possessed a slightly eccentric nucleus typical of a normal mast cell, and appeared throughout the connective tissue trabeculae and in the sinusoids of the red pulp. Some of the proliferating mast cells were intermixed with lymphocytes in the white pulp but, in general, the germinal centers were spared. As assessed immunohistochemically, most, if not all, of the mast cells in the spleen of this mastocytosis mouse expressed mMCP-2, mMCP-5, mMCP-6, mMCP-7, and mMC-CPA (Figure 1). When nonfractionated bone marrow cells from this primary mastocytosis mouse were injected into 4 sublethally irradiated mice, all recipient animals became moribund within 4 weeks. The recipient mice developed a systemic mastocytosis similar to that of the original animals, indicating that the disease was readily transferable.

Derivation of Mast Cell and Lymphoblastoid Cell Lines from the Primary Mastocytosis Mice

The V1, V8, and V9 lymphoblastoid cell lines were derived by culturing bone marrow cells from the primary animals

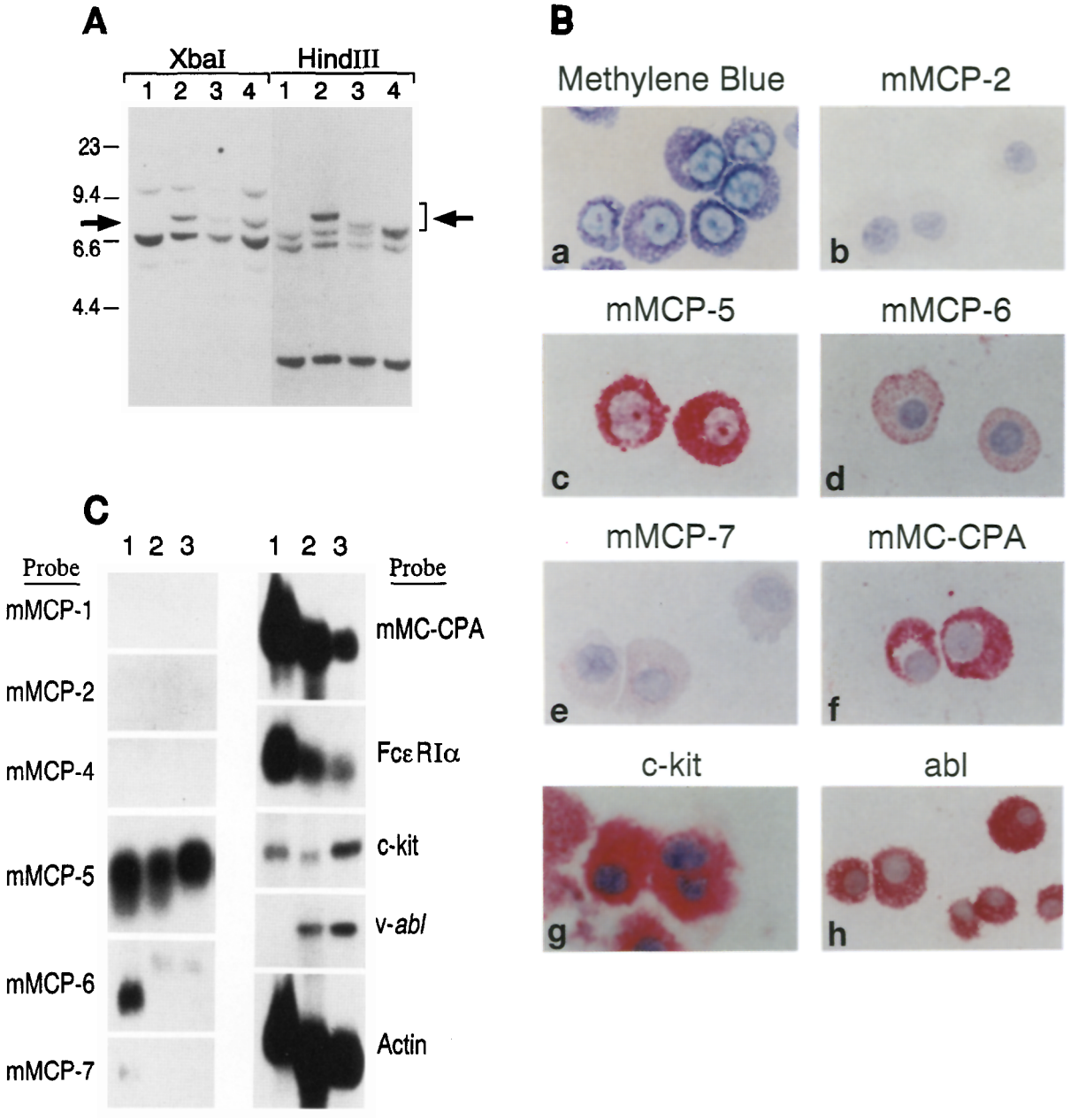


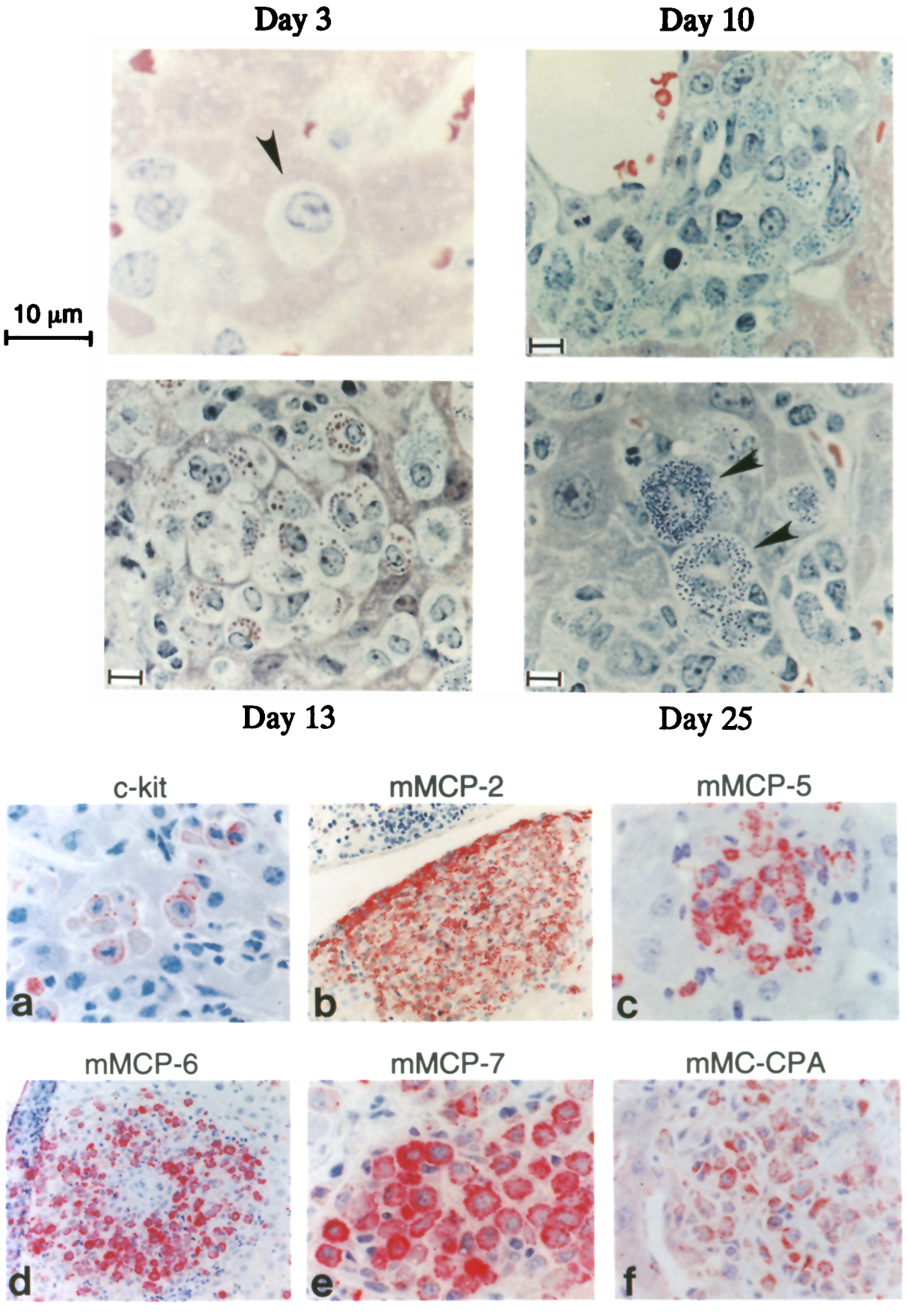
Figure 2. Characterization of the V3-MC and V7-MC Lines

(A) A blot containing XbaI- or HindIII-digested genomic DNA (5 μg/lane) from BALB/c mouse liver (lane 1) and from the V3-MCs (lane 2), V7-MCs (lane 3), and V8 lymphoblastoid cells (lane 4) was analyzed with the 3′ *abl* cDNA probe described in Experimental Procedures to assess proviral integration. XbaI cleaves once in each retroviral long terminal repeat, resulting in an ~7 kb pGD$^{v\text{-}abl}$-specific fragment if the entire provirus is integrated into the genome of the cell line. The arrow on the left indicates the ~7 kb DNA fragment that is not in liver but is in all three cell lines. HindIII cleaves once in the pGD$^{v\text{-}abl}$ retroviral vector. Thus, the number of provirus integrations in each cell line was determined by blot analysis of isolated genomic DNA digested with HindIII. The arrow and bracket on the right indicate the specific pGD$^{v\text{-}abl}$-derived fragment in each cell line. The HindIII results were confirmed using EcoRI which also cleaves pGD$^{v\text{-}abl}$ once (data not shown). The 9.4, 6.6, and 6.0 kb DNA fragments that are present in all four lanes are derived from the endogenous c-*abl* gene. Size markers, in kilobases, are indicated on the left.

(B) Cytospins of cultured V3-MCs were evaluated histochemically with methylene blue (a) or immunohistochemically with anti-mMCP-2 immunoglobulin (b), anti-mMCP-5 immunoglobulin (c), anti-mMCP-6 immunoglobulin (d), anti-mMCP-7 immunoglobulin (e), anti-mMC-CPA immunoglobulin (f), anti-c-*kit* immunoglobulin (g), or anti-*abl* immunoglobulin (h).

(C) Blots containing total RNA from BALB/c mBMMCs (lane 1), V3-MCs (lane 2), and V7-MCs (lane 3) were analyzed with gene-specific probes for mMCP-1, mMCP-2, mMCP-4, mMCP-5, mMCP-6, mMCP-7, mMC-CPA, FcεRIα, c-*kit*, *abl*, and actin.

that had mastocytosis and concomitant lymphoma. These three lymphoblastoid cell lines grew rapidly, and lines began to be established 2 weeks after initiation of the cultures. The V3-MC and V7-MC lines were derived from separate mice and were the only mast cell lines generated from the original 10 diseased animals. Unlike the lymphoblastoid cell lines, the mast cell lines did not rapidly proliferate until the fifth week of culture. Because the lym-


Day 3
Day 10
10 µm
Day 13
Day 25
c-kit
mMCP-2
mMCP-5
a
b
c
mMCP-6
mMCP-7
mMC-CPA
d
e
f

phoblastoid and mast cell lines were derived from separate mice, it could not be determined whether or not the two cell types were derived from a common v-*abl*-immortalized multipotential progenitor cell.

DNA blot analysis revealed that the V3-MC and V7-MC lines, as well as the V8 lymphoblastoid cell line, each contained a single integrated copy of the pGD$^{v\text{-}abl}$ provirus in their genomes at distinct sites (Figure 2A). As assessed by SDS–PAGE/immunoblot analysis, the V3-MC and V7-MC lines both expressed high levels of the 160 kDa v-*abl* protein (data not shown). V3-MCs and V7-MCs possess thin cytoplasmic projections (microvilli, microplicae, or both), a moderate amount of cytoplasm, and a single eccentric generally spherical or slightly indented nucleus (Figure 2B). As assessed histochemically, both cell lines are poorly granulated in vitro. They also contain low amounts of histamine (<100 ng/10^6 cells). As assessed immunohistochemically, cultured V3-MCs contain detectable levels of *abl*, c-*kit*, mMC-CPA, mMCP-5, and mMCP-6 proteins, but not mMCP-2 or mMCP-7 proteins (Figure 2B). The cultured V3-MCs stain less intensely with anti-mMCP-6 immunoglobulin than with anti-mMCP-5 immunoglobulin, and individual cells within the cultures differ in their intensity of staining by anti-mMCP-5 immunoglobulin. Nevertheless, when >300 cells in a culture were analyzed immunohistochemically, almost every cell contained some mMCP-5 and mMCP-6.

RNA blot analysis indicated that V3-MCs and V7-MCs contain high steady-state levels of the transcripts that encode c-*kit*, FcεRIα, mMC-CPA, and mMCP-5, but not mMCP-1, mMCP-2, mMCP-4, or mMCP-7 (Figure 2C). V3-MCs and V7-MCs also contain high steady-state levels of an ~8 kb transcript that was corecognized by *abl* (Figure 2C), *gag*, and *neo* cDNAs. This prominent transcript in the immortalized mast cell lines is therefore derived from the introduced provirus. The mMCP-6 gene-specific probe used in the RNA blot analysis hybridizes to a transcript in both cell lines that is less abundant than the mMCP-5 transcript. Its size is also 100–300 bp larger than the mMCP-6 transcript in BALB/c mBMMCs (Figure 2C). Except for the lack of the mMCP-7 transcript, the V3-MC and V7-MC lines phenotypically resemble mBMMCs derived by culturing BALB/c bone marrow cells for 3 weeks in IL-3-containing medium (Figure 2C). Because the steady-state level of the mMCP-7 transcript in mBMMCs decreases substantially with continued culture of these cells in medium containing IL-3 (McNeil et al., 1992b), the low steady-state level of the mMCP-7 transcript in the V3-MC and V7-MC lines is probably a consequence of their prolonged cultivation.

Adoptive Transfer with Histochemical and Immunohistochemical Characterization of Specific Tissues in the V3 Mastocytosis Mouse

Because the V3-MC line is indistinguishable from the V7-MC line in terms of its morphology, histochemistry, and protease phenotype, the V3-MC line was arbitrarily chosen for subsequent adoptive transfer experiments. In preliminary experiments, it was found that a reproducible systemic mastocytosis develops in normal nonirradiated syngeneic recipients after intravenous injection of V3-MC. Following adoptive transfer of V3-MCs into naive 4- to 6-week-old BALB/c mice, different tissues from replicate animals were evaluated over an interval of ~4 weeks for changes in their granule maturity and protease phenotype. With time, all V3 mastocytosis mice exhibited lethargy, dyspnea, a distended abdomen, and, eventually, death. Their spleens increased ~15-fold in weight and had a mottled appearance with tan infiltrates. The spleens contained ~200 mast cells/mm^2 2 weeks after adoptive transfer of V3-MCs, and numerous granulated V3-MCs were observed in mitosis in the spleen and liver. The livers increased ~10-fold in weight during the same period, and eventually had multiple punctate pale tan areas. In addition to the splenic and hepatic involvement, small nodules, often macroscopic, studded the mesentery and serosal surfaces of the intestine and the pleural surfaces of the lung. Lymph nodes were enlarged and tan. Despite the aggressive mastocytosis that developed in the liver, spleen, lung, stomach, and intestine of V3-MC-treated BALB/c mice, increased numbers of mast cells were not detected in the ear skin of the diseased animals.

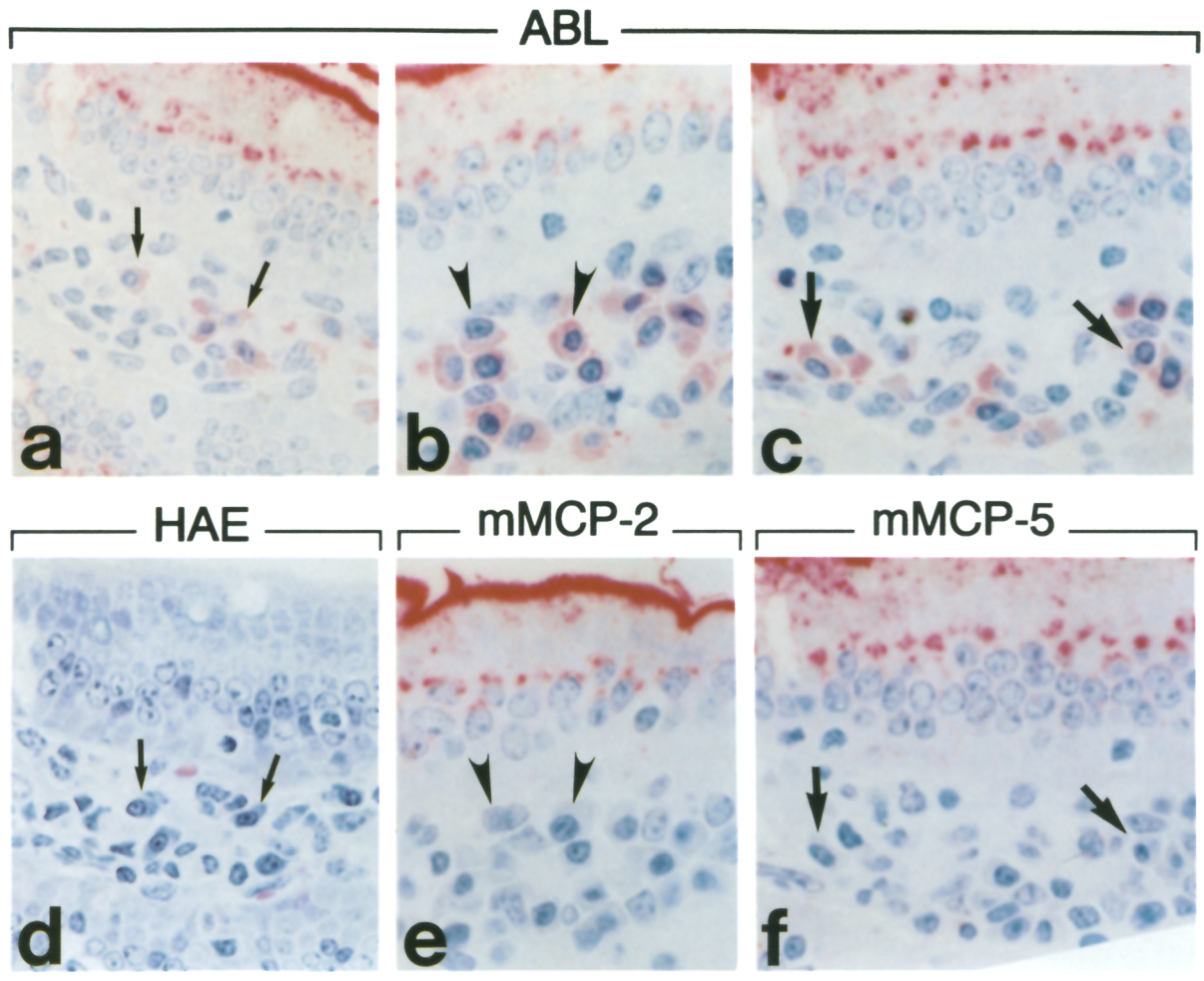
Because V3-MCs first populated the liver, kinetic histochemical studies were focused on this organ. Individual mononuclear cells with clear agranular cytoplasm appeared in the hepatic sinusoids 3 days after the injection of V3-MCs and in the epithelium among the hepatocytes (Figure 3). By 1 week, the V3-MCs in the liver were more numerous and they had small blue granules when stained by methylene blue or hematoxylin, azure II, and eosin-Y (HAE). At day 10, colonies of V3-MCs several millimeters in diameter were detected that often infiltrated and displaced the cords of hepatocytes (Figure 3). The V3-MCs in these colonies possessed metachromatic granules. Im-

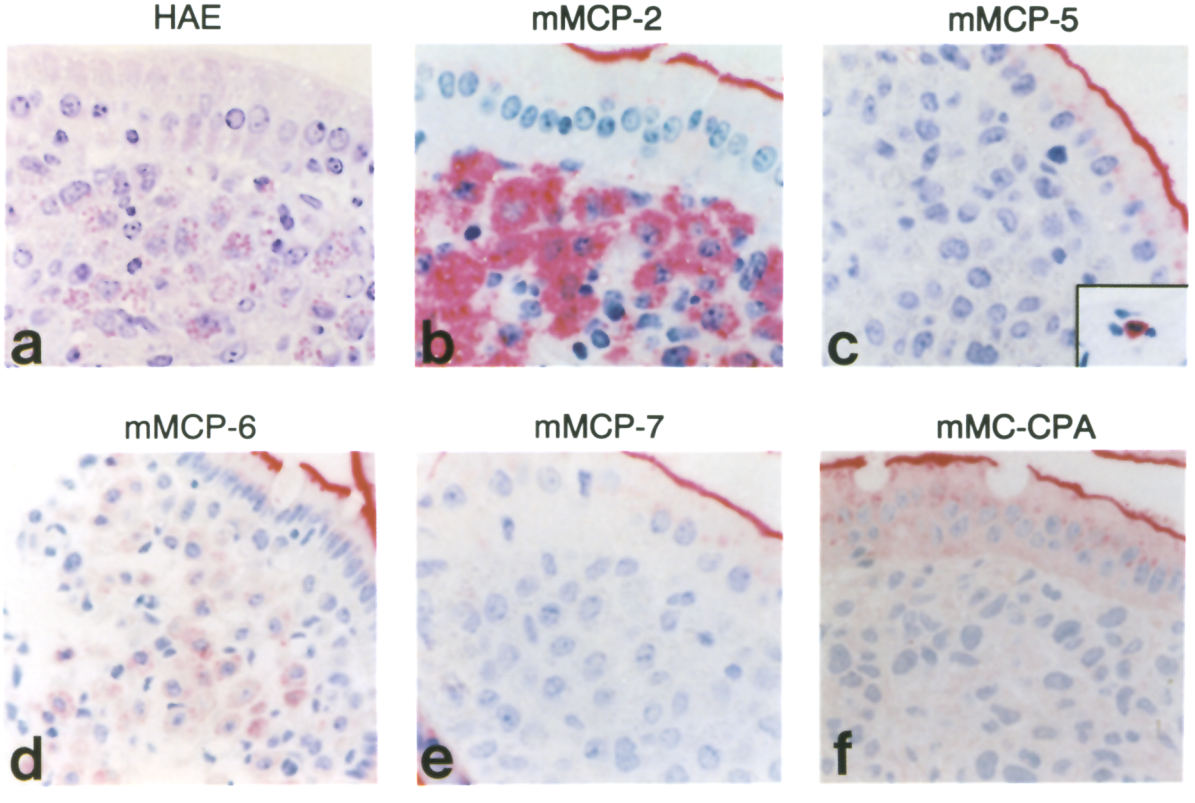
Figure 3. HAE Staining of V3-MCs in the Livers of Mastocytosis Mice

V3-MCs were adoptively transferred into nonirradiated BALB/c mice and the animals were analyzed 3, 10, 13, and 25 days later. Sections of liver were stained with HAE to evaluate the number of mast cells in this tissue, as well as the extent of their granule maturation. The arrow in the day 3 specimen indicates a nongranulated cell that has infiltrated the liver. Since subsequent studies revealed that this agranular cell type expresses *abl* protein, the cell most likely is a V3-MC. The two arrows in the day 25 specimen indicate histochemically mature mast cells. The brackets in the panels correspond to 10 μm.

Figure 4. Immunohistochemistry of the Livers of V3 Mastocytosis Mice

The livers in the depicted analyses were removed 2–3 weeks after V3-MCs were adoptively transferred into nonirradiated BALB/c mice. Sections were prepared and evaluated immunohistochemically with anti-c-*kit* immunoglobulin (a), anti-mMCP-2 immunoglobulin (b), anti-mMCP-5 immunoglobulin (c), anti-mMCP-6 immunoglobulin (d), anti-mMCP-7 immunoglobulin (e), or anti-mMC-CPA immunoglobulin (f). (b) and (d) are at low magnification.


ABL
a
b
c
HAE
mMCP-2
mMCP-5
d
e
f


HAE
mMCP-2
mMCP-5
a
b
c
mMCP-6
mMCP-7
mMC-CPA
d
e
f

mature agranular cells were still present in the sinusoids and liver cords at day 13, but most of the V3-MCs had developed large eosin-Y$^+$ granules. At days 10–16, when the mast cells exhibited substantial histochemical maturation, numerous eosinophils were also present among the mast cell infiltrates in the liver (data not shown). By day 25 (Figure 3), many of the V3-MCs in the liver had dense granules that were stained better by azure II. These mast cells histochemically resembled the mature indigenous mast cells in the spleen. Accompanying the massive infiltrate of mast cells in the liver of the day 25 mastocytosis mouse were areas infiltrated by polymorphonuclear leukocytes and macrophages, and bordered by fibroblasts. However, there was a marked decrease in the number of eosinophils associated with this late stage of the disease.

While undergoing histochemical maturation, the granules of the V3-MCs in the liver expressed increasing amounts of mast cell proteases, some of which were not detected in the cultured cells. As assessed by immunohistochemistry, the V3-MCs that populated the liver at day 3 expressed mMCP-5, mMCP-6, mMC-CPA, c-*kit*, and *abl* protein, but not mMCP-2 or mMCP-7 protein (data not shown). By week 2, the V3-MCs in the liver contained even more mMCP-5, mMCP-6, and mMC-CPA protein and now also contained mMCP-2 and mMCP-7 protein (Figure 4). There was no apparent diminution in the level of these proteases in the liver-localized V3-MCs during the late stages of the mastocytosis (days 28–35).

The V3-MCs that populated the spleen and small intestine also underwent a differentiation process. In the spleen, the adoptively transferred V3-MCs filled the red pulp and connective tissue trabeculae, impinging on the white pulp, and they eventually infiltrated the germinal centers of the lymphoid follicles. These splenic V3-MCs underwent developmental and maturational changes similar to the changes exhibited by V3-MCs in the liver, expressing mMCP-2, mMCP-5, mMCP-6, mMCP-7, and mMC-CPA in their granules. For comparison, mast cells in the normal BALB/c mouse spleen are rare (9–13 mast cells/5.2 mm^2 transverse section, n = 6); but, as assessed immunohistochemically (n = 3), virtually all of the mast cells in the normal spleen expressed mMCP-5, mMCP-6, mMCP-7, and mMC-CPA protein. Approximately 20% of the mast cells in the spleen of the normal BALB/c mouse also expressed mMCP-2 protein.

In the small intestine, mast cells accumulated predominantly in the lamina propria of the mucosa. There were also small numbers of mast cells within the smooth muscle layers and on the serosal surfaces of this organ. Cells that morphologically resembled V3-MCs and reacted with the anti-*abl* immunoglobulin were detected in the lamina propria 6 days after adoptive transfer (Figure 5). These cells lacked metachromatic granules and did not react with anti-mMCP-2 immunoglobulin or anti-mMCP-5 immunoglobulin. Nevertheless, 2–3 weeks after adoptive transfer, the V3-MCs in the lamina propria and between the epithelial cells in the mucosa of the small intestine possessed metachromatic granules (Figure 6). Thus, as in the liver and spleen, the V3-MCs that populated the intestine underwent granule maturation. Unlike in the liver (see Figure 4) or spleen, the V3-MCs in the intestine at week 2 preferentially contained mMCP-2 protein with little, if any, mMCP-5, mMCP-6, mMCP-7, or mMC-CPA protein (Figure 6). In contrast, the V3-MCs and the indigenous mast cells in the smooth muscle and on the serosal surface of the intestine expressed predominantly mMCP-5 (Figure 6), mMCP-7, and mMC-CPA, rather than mMCP-2.

RNA Blot Analysis of Tissues of the V3 Mastocytosis Mouse

As assessed by RNA blot analysis, no mast cell protease or *abl* transcript was detected in abundance in the liver, spleen, or small intestine of the normal BALB/c mouse (data not shown). However, 2–3 weeks after adoptive transfer of V3-MCs, the liver (Figure 7) and spleen (data not shown) of the V3 mastocytosis mouse contained high steady-state levels of the ~8 kb v-*abl* transcript and every mast cell protease transcript that has so far been cloned. In contrast, the only abundantly expressed transcripts in the intestine of the 2–3 week V3 mastocytosis mouse were those that encode v-*abl*, mMCP-1, and mMCP-2 (Figure 7). Similar findings were obtained when four of the granule proteases were assessed in three independent subcloned lines of V3-MC (designated V3-MC-I, V3-MC-II, and V3-MC-III). After adoptive transfer into separate mice, these subcloned V3-MCs populated and proliferated in the liver and intestine. In the liver, they expressed high steady-state levels of the mMCP-1, mMCP-2, mMCP-4, and mMCP-5 transcripts at day 17 (data not shown). However, in the intestine, they expressed high steady-state levels of the mMCP-1 and mMCP-2 transcripts but not the mMCP-4 and mMCP-5 transcripts at day 17 (data not shown). The RNA blot and immunohistochemical data are summarized in Table 1.

Figure 5. Histochemical and Immunohistochemical Analysis of the Small Intestines of V3 Mastocytosis Mice 6 Days after Exposure to V3-MCs

Serial sections (a and d; b and e; c and f) of the small intestinal mucosa 6 days after adoptive transfer of V3-MCs were evaluated histochemically with HAE (d) or immunohistochemically with anti-*abl* immunoglobulin (a, b, and c), anti-mMCP-2 immunoglobulin (e), or anti-mMCP-5 immunoglobulin (f). The arrows indicate cells expressing *abl* protein (a, b, and c) whose granules do not stain with HAE (d). These *abl*-positive cells also are not recognized by anti-mMCP-2 immunoglobulin (e) or anti-mMCP-5 immunoglobulin (f).

Figure 6. Histochemical and Immunohistochemical Analysis of the Small Intestines of V3 Mastocytosis Mice 2 Weeks After Exposure to V3-MCs

Sections of intestine were prepared and evaluated histochemically with HAE (a) or immunohistochemically with anti-mMCP-2 immunoglobulin (b), anti-mMCP-5 immunoglobulin (c), anti-mMCP-6 immunoglobulin (d), anti-mMCP-7 immunoglobulin (e), or anti-mMC-CPA immunoglobulin (f). The submucosa of the depicted V3 mastocytosis mouse contains very few mast cells. However, the insert in the bottom right of (c) is a mast cell in the submucosa of the same section that is stained by anti-mMCP-5 immunoglobulin.

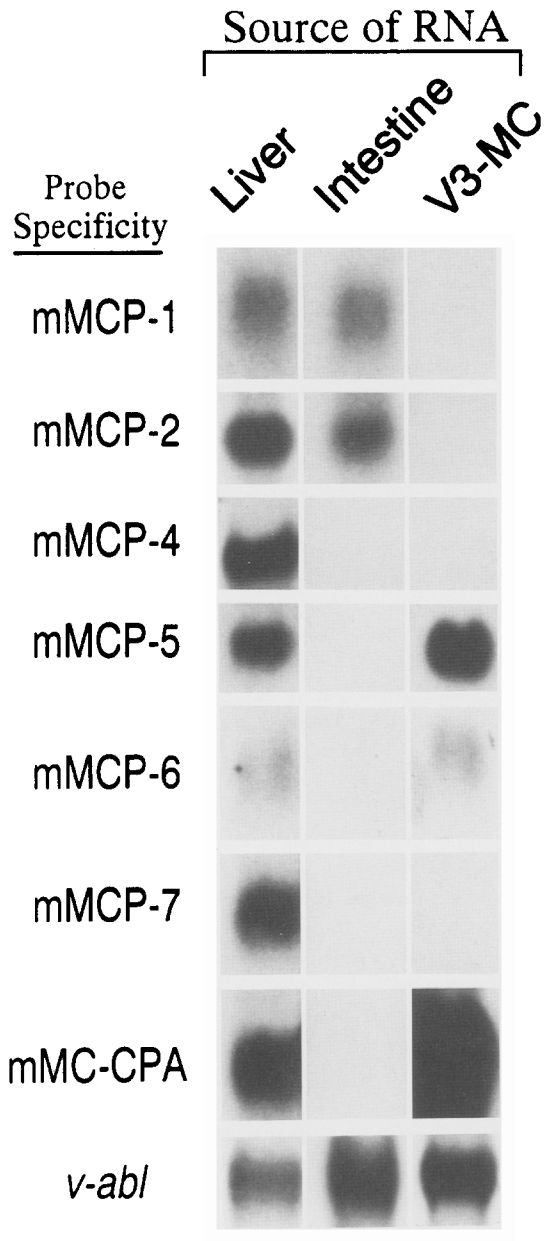


Figure 7. RNA Blot Analysis of Mast Cell Protease Transcripts in the Livers and Small Intestines of V3 Mastocytosis Mice

Blots containing total RNA from cultured V3-MCs, and from the intestine and liver of a V3 mastocytosis mouse 2 weeks after adoptive transfer were analyzed with gene-specific probes for mMCP-1, mMCP-2, mMCP-4, mMCP-5, mMCP-6, mMCP-7, mMC-CPA, and *abl*.

Discussion

The lineage-restricted mast cell progenitor line, V3-MC, was derived from a mouse that had developed systemic mastocytosis after transplantation of bone marrow cells transduced with v-*abl* (Figure 1). Several characteristics of V3-MCs make this cell line a unique reagent for studying mast cell differentiation in vivo. First, it does not release infectious v-*abl* virions. As a result, all developmentally regulated changes are due to changes in the injected cell line itself, rather than infection of new targets after intravenous injection. Second, by virtue of its single integrated provirus, the V3-MC line appears to have been derived from a single clone. In further support of its clonal derivation, the protease expression pattern of the V3-MC line has not varied during the year that it has been cultured. Third, V3-MCs share many phenotypic characteristics with immature mast cell precursors and can be induced to differentiate in vivo.

Although adoptive transfer experiments in mast cell–deficient mice have shown that mast cell precursors are present in the peripheral blood (Sonoda et al., 1982), this population has not been purified. The best characterized immature mast cell is the IL-3-dependent mBMMC, and it is this population with which V3-MCs share many phenotypic characteristics. In both cell types, histamine content is less than 0.5 pg/cell and the neutral proteases expressed are mMCP-5, mMCP-6, and mMC-CPA. In mBMMCs and V3-MCs, the steady-state levels of the mMCP-1, mMCP-2, and mMCP-4 transcripts are below detection. One difference between the protease expression pattern of mBMMCs and V3-MCs is that mMCP-7 is expressed only transiently in the former (McNeil et al., 1992b). As the levels of this transcript fall with prolonged culture, its absence in V3-MCs may reflect the extended period that this line has been maintained in vitro.

When the V3-MC line is adoptively transferred into BALB/c mice, a reproducible aggressive mastocytosis develops in the liver, spleen, and small intestine. The skin appears to be spared even at week 4, thereby emphasizing the importance of tissue-dependent homing of immature progenitor cells. The observation that adoptively transferred V3-MCs populate the spleen but not the skin of the BALB/c mouse is similar to findings obtained when mast cell–deficient W/W^v mice are reconstituted with mouse bone marrow cells from their normal littermates (Kitamura et al., 1978; Du et al., 1994). No significant increase in the number of cutaneous mast cells was observed in the reconstituted W/W^v mouse 5 weeks after adoptive transfer. Moreover, even 15 weeks after adoptive transfer, the density of mast cells in the skin of the reconstituted W/W^v mouse does not approach that found in its normal littermate. Thus, mast cell repopulation of skin is a slow process in mast cell–deficient W/W^v mice. The V3-MC data suggest that this also is the case in mast cell–sufficient BALB/c mice. A number of factors could account for the failure of V3-MCs to populate the skin. Homing of committed progenitors into the skin may preferentially occur at an earlier timepoint in ontogeny. V3-MCs may be too far along in their differentiation pathway or they may lack some critical adhesion factor. Alternatively, the skin may have a mechanism(s) that effectively regulates the number of mature and progenitor mast cells in the tissue.

After transfer, *abl*-expressing agranulated cells are detectable in both the liver at day 3 and small intestine lamina propria at day 6. In the liver, these cells are found together with granulated V3-MCs, whereas in the small intestine lamina propria, the agranulated cells are present before any granulated cells are detected. Because cells that have no granules account for approximately 5% of the V3-MCs in vitro, it is possible that this population of agranulated

Table 1. Protease Expression of Cultured V3-MCs and Tissue-Localized V3-MCs

	Cultured[b] V3-MC	Liver[a] V3-MC (day 3)	Liver[b] V3-MC (week 2–3)	Intestinal[a] V3-MC (day 6)	Intestinal[b] V3-MC (week 2–3)
mMCP-1	–	ND	+	ND	+
mMCP-2	–	–	+	–	+
mMCP-4	–	ND	+	ND	–
mMCP-5	+	+	+	–	–
mMCP-6	+(weak)	+	+	–	–
mMCP-7	–	–	+	–	–
mMC-CPA	+	+	+	–	–

[a] Immunohistochemical data; ND, not determined.
[b] Immunohistochemical and RNA blot data.

cells preferentially home to these tissues. Alternatively, the granulated V3-MCs may degranulate or catabolize their granular proteases before or shortly after their appearance in these organs.

With time, the V3-MCs that proliferate in the liver (Figure 3), spleen, and intestine (Figures 5 and 6) become increasingly metachromatic. This finding indicates either increased granule storage of serglycin proteoglycans or a change in the type of glycosaminoglycan synthesized onto the granule-localized proteoglycan peptide core. As assessed by RNA blot and immunohistochemical analyses, V3-MCs both differentiate and mature in tissues. V3-MCs that are present in the liver (Figures 4 and 7) and spleen of the 2- to 3-week-old mastocytosis mouse acquire expression of mMCP-1, mMCP-2, mMCP-4, and mMCP-7, while maintaining expression of the three granule proteases expressed by the starting cultured cells (mMCP-5, mMCP-6 and mMC-CPA) (Figure 2). This protease expression pattern is similar to that of a subpopulation of mast cells found in the spleen of normal BALB/c mice. The paucity of indigenous mast cells in the normal liver has precluded their phenotyping.

Numerous cytokines have been identified that influence protease expression in cultured mouse mast cells (Gurish et al., 1992a; Ghildyal et al., 1992a, 1992b, 1993). mBMMCs cultured first in the presence of IL-3 and then with the combination of IL-9 and c-*kit* ligand (Eklund et al., 1993) resemble the V3-MCs in the liver and spleen in expressing every known mast cell neutral protease. The present results are a novel description of mast cells in vivo that express all of the cloned mast cell proteases. It is possible that the protease phenotypes of the liver and spleen V3-MCs are induced by a similar combination of mast cell–regulatory cytokines.

The V3-MCs in the small intestine lamina propria at week 2–3 have developed a phenotype distinct from that found in liver and spleen at this time. The V3-MCs that initially populate the small intestine were not recognized by any of the mast cell protease-specific antibodies (Figure 5). However, by week 2–3, V3-MCs in the lamina propria express principally mMCP-2 and little or no mMCP-5, mMCP-6, mMCP-7, or mMC-CPA (Figure 6). Since the steady-state levels of the mMCP-1 and mMCP-2 transcripts are comparably increased in the intestine of the mastocytosis mouse (Figure 7), it is presumed that the V3-MCs in the intestinal lamina propria also express mMCP-1 protein. Mast cells are rare inhabitants of the normal intestinal lamina propria and even rarer in the epithelium. Nevertheless, the mucosal mast cells that are found at both sites express mMCP-2 (Friend et al., 1995). Upon helminth infection, they become more abundant and continue to preferentially express mMCP-1 (Le Trong et al., 1989; Huntley et al., 1990; Ghildyal et al., 1992b) and mMCP-2 (Serafin et al., 1990; Ghildyal et al., 1992b; 1993). Therefore, the V3-MCs that reside in the small intestine mucosa not only are phenotypically distinct from the V3-MCs in the liver and spleen but also take on the phenotypic characteristics of indigenous mucosal mast cells. Although there were considerably fewer numbers of mast cells in the intestinal submucosa of the week 2 or 3 mastocytosis mouse compared with the numbers in the lamina propria, these cells express mMCP-5, mMCP-6, mMCP-7, and mMC-CPA but little or no mMCP-2. This granule protease phenotype is identical to the indigenous mast cells that reside in the submucosa. Thus, the dichotomy of phenotypes found in the mouse small intestine has been preserved in the phenotypes of the V3-MCs that populate these two regions of the intestine.

The studies of Kitamura and coworkers on the reconstitution of mice with bone marrow cells or with mBMMCs (Kitamura et al., 1977, 1978; Nakano et al., 1985) indicated that mast cells differ from other hematopoietic-derived cells in that they do not circulate in blood as mature cells. These studies also raised the possibility that mast cell heterogeneity was the result of the tissue-specific differentiation of a common progenitor. In contrast, recent studies of human mast cell development (reviewed by Irani and Schwartz, 1994) led to the proposal that different populations of mast cells arise from distinct progenitor cells. The current approach with the tissue-responsive V3-MCs provides an opportunity to examine the pleiotropism of committed mast cell progenitors in vivo in a mast cell–sufficient mouse. Our results argue against mast cell heterogeneity deriving from multiple progenitor populations. Instead, our data indicate that V3-MCs are capable of homing to several different tissues, where they undergo phenotypic changes, becoming indistinguishable from the indigenous mast cells. These changes involve both the acquisition and loss of specific granule-localized neutral proteases. In vitro studies have shown that different combinations of cytokines can modulate the protease expression patterns of cultured mBMMCs. Although the current studies do not

allow us to identify the mechanisms accounting for the tissue-specific mast cell phenotypes of the V3-MCs, it appears that cytokines or other tissue-specific factors influence the final steps of mast cell differentiation.

Experimental Procedures

Derivation and Use of a v-*abl*-Transformed Mast Cell Line to Create Systemic Mastocytosis in Mice

The pGD$^{v\text{-}abl}$ retroviral DNA construct used to transfect Bosc 23 cells has been previously described (Scott et al., 1991), as well as the cell transfection and cocultivation procedures (Pear et al., 1993). Parallel experiments showed that the v-*abl* retroviral titer was ~2 × 10^6 focus-forming U/ml of retroviral supernatant. As assessed by the BAG cell assay (Pear et al., 1993, and references therein), replication-competent virus was not present. The donor mice were 4- to 6-week-old male BALB/c mice (Jackson Laboratories) that had been pretreated with 5-fluorouracil (100 mg/g body wt). The recipient mice (4- to 6-week-old BALB/c female mice that had received 900 cGy [two doses separated by 3 hr]) were injected intravenously with 0.5 to 1.0 × 10^6 cells/mouse. Blood was isolated from the mice 3 weeks after marrow transplantation to determine white blood cell count indices and to prepare DNA for PCR analysis, as previously described (Daley and Baltimore, 1988; Daley et al., 1990).

To derive growth factor–independent cell lines from the primary mastocytosis mice, bone marrow was obtained at the time of necropsy and cultured in enriched medium (Dulbecco's modified Eagle's medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 50 mM 2-mercaptoethanol, and 20% fetal calf serum) for up to 3 months. Two growth factor–independent mast cells lines (V3-MC and V7-MC) and three growth factor–independent nonmast cell lines (V1, V8, and V9) were derived from different primary animals. As assessed by fluorescein-activated cell sorter analyses, the V1, V8, and V9 cell lines were recognized by an antibody to B220 but not by antibodies to Thy1.2, IgM, Gr-1, or Mac-1 (data not shown). Because these findings suggested that the V1, V8, and V9 cell lines were of pre-B cell origin, they were not studied further. To ensure that the V3-MCs that populate the liver represent the same population of cells in the culture that populates the intestine, three independent subcloned V3-MC lines (designated V3-MC-I, V3-MC-II, and V3-MC-III) were obtained by limiting dilution.

V7-MCs and V3-MCs were maintained in vitro in enriched medium and were passaged twice each week. The histamine content of cultured V3-MCs was determined by radioimmunoassay (McBride et al., 1988). For induction of systemic mastocytosis by adoptive transfer, 1–4 × 10^6 V3-MCs in 0.2 ml of enriched medium were injected into the tail vein of nonirradiated syngeneic BALB/c mice. At various times after inoculation, the diseased mice were sacrificed and their tissues were processed immediately, as described below.

Histochemistry and Immunohistochemistry of V3-MCs and Various Tissues of the V3 Mastocytosis Mouse

For immunoalkaline phosphatase staining (Boenisch et al., 1989), cultured V3-MCs cytocentrifuged (5 min; 28 × g) onto glass slides or isolated tissues from V3 mastocytosis mice were fixed for 4 hr at room temperature in 4% paraformaldehyde and 0.1 M sodium phosphate (pH 7.6) (Beckstead et al., 1981). Preparations were washed twice with phosphate-buffered saline (PBS) containing 2% dimethyl sulfoxide, and then were suspended in 50 mM NH_4Cl overnight at 4°C. Dehydration and embedding of the specimens were carried out in accordance with the JB-4 embedding kit instructions from Polysciences, Incorporated. After 1–2 days of hardening, 1.5 μm thick sections of fixed and embedded tissue were cut on a Reichert–Jung Supracut microtome (Leica Corporation) using glass knives and picked up on glass slides. The slides were incubated sequentially for 15 min at 37°C in 2 mM $CaCl_2$ containing 0.25% trypsin, for 15 min at room temperature in PBS containing 0.05% Tween 20 and 0.1% bovine serum albumin, for 30 min at 37°C in PBS containing 0.05% Tween 20 and 4% normal goat serum, and overnight at 4°C in 4% normal goat serum containing affinity-purified rabbit anti-mast cell protease immunoglobulin, rat anti-mouse c-*kit* immunoglobulin (Pharmingen), or mouse anti-*abl* immunoglobulin (Schiff-Maker et al., 1986). Rabbit anti-mMCP-2 immunoglobulin (Ghildyal et al., 1993), anti-mMCP-5 immunoglobulin (McNeil et al., 1992a), anti-MCP-6 immunoglobulin (N. G., unpublished data), anti-mMCP-7 immunoglobulin (Ghildyal et al., 1994), and anti-mMC-CPA immunoglobulin (Gurish et al., 1992b) were obtained against synthetic peptides that correspond to residues 56–71, 146–162, 160–172, 160–172, and 146–157 in the respective protease. The anti-*abl* mouse monoclonal antibody (designated 24-21) used in this study reacts equally well with c-*abl* and v-*abl* (Schiff-Maker et al., 1986). The samples were washed, incubated for 40 min at room temperature in buffer containing biotin-labeled goat anti-rabbit IgG, goat anti-rat IgG, or goat anti-mouse IgG. Each was then washed twice in 0.1% bovine serum albumin and 0.05% Tween 20 in PBS, incubated for 40 min at room temperature in Vectastain ABC-AP reagent (Vector Laboratories), and then incubated for 15 min in the dark at room temperature in an alkaline phosphatase substrate solution. The cytochemical data presented in Results were derived from 24 separate V3 mastocytosis mice. Controls consisted of sections of tissue from four different mice treated with nonimmune IgG (Endogen, Incorporated) or without primary antibody. Cytospins of V3-MCs or tissue sections were counterstained with Gill's hematoxylin in 20% ethylene glycol, and then coverslips with Immu-Mount (Shandon) were applied.

For histological examination, cultured V3-MCs or serial 1.5 μm thick glycolmethacrylate sections of various tissues from the normal BALB/c mouse or the V3 mastocytosis mouse were placed on coverslips or slides, air dried, and stained for 20 s in a 5% ethanolic solution of methylene blue (Matin et al., 1992). Alternatively, sections were incubated sequentially with double-strength hematoxylin for 2 min, 1% aqueous eosin Y for 15 min, azure II for 1 min, and ethylene glycol monomethyl ether for 5 s (Beckstead et al., 1981).

For quantitating the mast cells in a tissue, areas were traced from paraformaldehyde-fixed glycolmethacrylate-embedded blocks of tissue, sectioned at 1.5 μm thickness, and stained with methylene blue, toluidine blue, HAE, or with the immunoalkaline phosphatase procedure. Using a drawing tube attachment to an optical Leitz microscope, the areas traced were then measured on a computer-controlled digitizing board employing a Sigma Scan digitizing program (Jandel Scientific). Mast cells were counted on a Dialux 20 optical microscope with an oil immersion 50× objective, and the results were expressed as the number of mast cells/mm^2. The number of alkaline phosphatase immunoreactive mast cells was determined in a similar manner. Although qualitative differences were observed among the mast cells, the criteria for a V3-MC being scored positive for the expression of a particular protease required at least three cytoplasmic granules stained with the protease-specific antibody. Data were expressed as the number of immunoreactive cells/mm^2.

RNA Blot Analysis

Tissues were surgically excised, immediately frozen in liquid nitrogen, ground in liquid nitrogen with a mortar and pestle, and then homogenized briefly in 4 M guanidinium thiocyanate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol, and 25 mM sodium citrate (Stevens et al., 1994). Total RNA was isolated using the method of Chomczynski and Sacchi (1987) from the tissues of V3 mastocytosis mice, from cultured V3-MCs, and from control BALB/c mBMMCs (Razin et al., 1984). The resulting RNA samples were denatured in formaldehyde/formamide, electrophoresed in 1.3% formaldehyde–agarose gels, and transferred to MagnaGraph membranes (Micron Separations, Incorporated) (Thomas, 1980). The blots were then incubated overnight at 43°C in hybridization buffer containing 5× SSPE, 5× Denhardt's buffer, 0.1% SDS, 100 μg/ml salmon sperm DNA, and a radiolabeled gene-specific probe for mMCP-1 (Le Trong et al., 1989; Huang et al., 1991; Ghildyal et al., 1992b), mMCP-2 (Serafin et al., 1990), mMCP-4 (Serafin et al., 1991; Ghildyal et al., 1992b), mMCP-5 (McNeil et al., 1991), mMCP-6 (Reynolds et al., 1991), mMCP-7 (McNeil et al., 1992b), mMC-CPA (Reynolds et al., 1989), c-*kit* (Flanagan and Leder, 1990), or β-actin (Spiegelman et al., 1983). The RNA blots were washed at 55°C–60°C in 15 mM NaCl, 1.5 mM sodium citrate, and 0.1% SDS and analyzed by autoradiography, by Betascope (Betagen), or by both. Blots were stripped and reanalyzed with other gene-specific probes. The *abl* probe used in this study corresponds to the 3′ end of the gene. It was derived by PCR amplification of residues 3152 to 4582 of pGD$^{v\text{-}abl}$ (Scott et al., 1991). The primers used for this PCR were 5′-TGGGGCAAGGGACACAGAGT-3′ and 5′-CACAGGCAGTGAGGAGAGGT-3′. Be-

cause of their near identity, the obtained *abl* probe will recognize the ~5-kb c-*abl* transcript and the ~8-kb pGD$^{v\text{-}abl}$-derived transcript equally well. However, the two related transcripts can be distinguished from one another by their different sizes in a RNA blot analysis. The *gag* probe used in this study was derived by PCR amplification of residues 700–1308 of pGD$^{v\text{-}abl}$ (Scott et al., 1991). The primers used for this latter PCR were 5′-CGGTAGATGTCAAGAAGAGA-3′ and 5′-TAAAGGTCAGAAGAGGAGAA-3′. An ~900-bp DNA probe (*neo*) was obtained from the neomycin-resistance gene by digesting pMC1*neo* (Stratagene) with EcoRI and BamHI. The *gag*, *abl*, and *neo* probes recognize the same transcript in pGD$^{v\text{-}abl}$-immortalized cells.

Acknowledgments

This work was supported by grants AI-22346, AI-22531, AI-23483, AI-31599, AR-07530, AR-36308, RR-05950, HL-48598, and HL-36110 from the National Institutes of Health, and by a grant from the Hyde and Watson Foundation. W. S. P. was supported by a physician postdoctoral fellowship from the Howard Hughes Medical Institute. We thank Mr. B. Soffer for technical assistance, Dr. J. Flanagan (Harvard Medical School) for the c-*kit* cDNA, and Dr. N. Rosenberg (Tufts Medical School) for the 24-21 anti-*abl* monoclonal antibody.

Received March 15, 1995; revised June 9, 1995.

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